# FINAL REPORT

Grant#: N00014-96-1-0619

PRINCIPAL INVESTIGATOR: Tracy Romano, PhD

INSTITUTION: Texas A&M University

GRANT TITLE: Investigation of Immune Function in Naval Marine Mammals

AWARD PERIOD: 1 June 1996 - 31 October 1999

OBJECTIVE: To generate cetacean-specific immunological reagents in order to investigate immune function in Navy marine mammals.

APPROACH: We have generated molecular reagents and antibodies specifically to cetacean lymphocyte surface proteins. The approach utilized molecular biology technology and monoclonal and polyclonal antibody production. Primarily, our efforts have focused on the cell surface molecule CD4, which plays a key role in the immune response. CD4 was cloned from a white whale, Delphinapterus leucas, thymus cDNA library using the polymerase chain reaction and cDNA library screening. Polyclonal antibodies were raised to different peptide fragments of the whale CD4 molecule as well as the expressed CD4 protein, and monoclonal antibodies were generated to the expressed CD4 protein. The antibodies have been characterized by ELISA, Western blot analysis, and immunofluorescence.

ACCOMPLISHMENTS: Our efforts over the past 36 months of ONR support have resulted in multiple accomplishments. These accomplishments include:
1) *The characterization of whale CD4 at the molecular level.* White whale (*Delphinapterus leucas*) CD4 was cloned using the polymerase chain reaction and by screening a whale cDNA thymus library. Clones were sequenced, analyzed, and compared to CD4 of other mammals including human.
2) *The generation of polyclonal anti-peptide antibodies against whale CD4.* The whale CD4 sequence was analyzed to look at antigenic determinants and secondary structure in order to design peptides for anti-peptide antibody production. Three different peptides were designed, coupled, and injected into rabbits for polyclonal antibody production and subsequently purified and characterized. These antibodies were useful in Western blot analysis.
3) *Expression and purification of the whale CD4 protein itself using a bacterial expression system and a baculovirus expression system.* Bacterial expression of whale CD4 was carried out using a GST fusion protein. The fusion protein was purified and injected into rabbits for polyclonal

antibody production. In order to obtain the native CD4 protein a baculovirus expression system was used to express whale CD4. Whale CD4 was grown up in large quantities and purified for subsequent antibody production.
4) *Methodology for the generation and screening of cetacean-specific monoclonal antibodies against whale CD4.* Whale CD4 protein was injected into mice for monoclonal antibody production. Since the end goal was to obtain an antibody that recognized native CD4 on the surface of cells, screening of the resultant hybridomas was carried out by flow cytometry analysis.
5) *Methodologies for characterization of generated antibodies.* These methodologies include the development of an ELISA cetacean cell assay, methodologies for carrying out immunofluorescence on cetacean lymphoid tissue, skin, and peripheral blood lymphocytes, and the preparation and labeling of cetacean cell lysates for Western blot and immunoprecipitation analysis.
6) *Discovery and characterization at the molecular level of a variant form of CD4 in the cetacean.* When cloning whale CD4 a variant form of CD4 was identified. Subsequent RT-PCR analysis and Northern blot analysis suggest the variant is not a cloning artifact.
7) *Collecting, preserving, and archiving cetacean lymphoid tissues and brain.* Tissues from the immune system and brain were collected from stranded cetaceans, from beluga whales taken during sanctioned hunts, and expired cetaceans at the Navy facility or aquariums and either frozen on dry ice or liquid nitrogen or fixed chemically to use in future experiments relating to the cetacean immune system.

CONCLUSIONS: Molecular characterization of CD4 in an aquatic mammal has provided information on the CD4 molecule itself and has provided some insight into adaptive evolutionary changes of the immune system. Although whale CD4 shares 64% and 51% identity with the human and mouse protein, it contains unique amino acid substitutions in the highly conserved cytoplasmic domain, 7 potential N-linked glycosylation sites, and lacks the cysteine pair in the V2 domain. These differences suggest that whale CD4 may have a different secondary structure, which may affect binding of class II and subsequent T cell activation, as well as binding of viral pathogens. The baculovirus expression system proved to be the most effective in producing the whale CD4 protein and generating antibodies against the native CD4 protein. With the CD4 molecular reagents we have generated we can begin to measure this important immune system protein in Navy marine mammals and monitor CD4 levels during sickness, before, during, and after deployment or transport, and before, during, and after environmental challenges such as acoustic challenges, thermal challenges, pollutants, introduction to a novel environment, changes in housing conditions, as well as routine health monitoring.

SIGNIFICANCE: The study and comparison of whale CD4 is significant in itself given the importance of this molecule in the immune response, its interaction with major histocompatibility class II molecules (of which cetaceans have a constitutive expression on T lymphocytes), and given its role in AIDS. Comparison of whale CD4 with other species has revealed interesting comparative information in regards to the evolution and adapation of the mammalian immune system.

The study of CD4 in cetaceans is valuable for clinical monitoring and health assessment of cetaceans kept under the Navy's care as well as for those in the wild. The CD4 reagents we presently have as well as an antibody that will recognize the native CD4 protein, (which we are currently screening for), will enable us to quantify baseline levels of CD4 in Navy dolphins and whales. Subsequently, we can monitor these levels and investigate how levels change during sickness, transport, routine and novel exercises, and environmental challenges such as thermal and acoustic challenges. We will also be able to label CD4-positive lymphocytes in the lymphoid organs of hunted, stranded, entangled (in fishing nets) and/or expired animals, and determine lymphocyte organization and interactions with postganglionic nerve fibers of the autonomic nervous system in primary and secondary lymphoid organs.

PATENT INFORMATION: No patents have been filed.

AWARD INFORMATION: Promoted to Assistant Research Scientist in the Dept. of Veterinary Anatomy/Public Health at Texas A&M University

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Romano, T.A., J.A. Olschowka, S.Y. Felten, V. Quaranta, S.H. Ridgway, and D.L. Felten. Immune response, stress, and evironment: Implications for cetaceans. In: *Cell and Molecular Biology of Marine Mammals*. C.J. Pfeiffer (ed). Krieger Publishing Co., Inc. (publication pending).

Romano, T.A., S.H. Ridgway, D.L. Felten, and V. Quaranta. 1998. Investigations of the cetacean immune system: Molecular cloning of beluga whale CD4. In: Society for Marine Mammalogy Abstracts/World Marine Mammal Conference, Monaco pp.116. (Abstract and presentation).

Romano, T.A., D.L. Felten, S.H. Ridgway, and V. Quaranta. 1998. Investigations of the cetacean immune system. In: XXII Reunion Internacional para el estudio de los Mamiferos Marinos Abstracts, Quintana Roo, Mexico, pp.52. (Invited abstract and presentation).

Romano, T.A., S.H. Ridgway, V. Quaranta, and D.L. Felten. 1997. Investigations of the cetacean immune system: Molecular cloning of lymphocyte CD4 in the beluga whale, *Delphinapterus leucas*. In:*Proceedings of the 1997 International Association for Aquatic Animal Medicine*. Vol. 28 (Abstract and presentation).

Plopper, G., J. Falk-Marzillier, S. Glaser, M. Fitchmun, G. Giannelli,T. Romano, J.C. Jones, and V. Quaranta. 1996. Changes in expression of monoclonal antibody epitopes on laminin-5R induced by cell contact. *J. Cell Science*. 109:1965-1973.

Romano, T.A., S.H. Ridgway, D.L. Felten, and V. Quaranta. 1996. The Development of Molecular Markers for Investigation of the Cetacean Immune System. In: *Proceedings of the 1996 International Association for Aquatic Animal Medicine*. Vol. 27 (Abstract and presentation).

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**13. ABSTRACT** *(Maximum 200 words)*

Marine mammals are used and maintained by the US Navy for military operations and research and development. Health maintenance is critical for optimal performance. However, very little is known about the immune system of marine mammals, especially the total aquatic cetaceans (dolphins, whales, and porpoises). This is due in part to obtaining access to animals and tissue samples as well as the lack of cetacean-specific reagents to carry out investigations of the immune system. To this end, we have cloned the gene for whale CD4, a cell surface protein present on T helper lymphocytes and important for the immune response. Whale CD4 shares 64% and 51% identity with the human and mouse protein. It contains unique amino acid substitutions in the highly conserved cytoplasmic domain, contains 7 potential N-linked glycosylation sites, and lacks the cysteine pair in the V2 domain. Bacterial and baculovirus expression systems were used to produce whale CD4 for subsequent injection into mice and/or rabbits for antibody production. Polyclonal antibodies have been used in Western blot analysis and hybridoma supernatants are currently being screened by flow cytometry. The whale-specific CD4 reagents we have generated will be used in health monitoring of Navy dolphins.

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